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Molecular Evidence for Genetic Heterogeneity and the Hybrid Origin of *Acrorumohra subreflexipinna* from Taiwan

HO-MING CHANG
Department of Life Science, National Taiwan Normal University, No.88, Ting-Chow Rd., Sec 4, Taipei 116, Taiwan
Division of Botany, Endemic Species Research Institute, No.1, Ming-Sheng E. Rd., Chi-Chi, Nantou 552, Taiwan
WEN-LIANG CHIOU
Division of Forest Biology, Taiwan Forestry Research Institute No. 53, Nanhai Rd., Taipei 100, Taiwan
JENN-CHE WANG*
Department of Life Science, National Taiwan Normal University No.88, Ting-Chow Rd., Sec 4, Taipei 116, Taiwan

ABSTRACT.—*Acrorumohra subreflexipinna*, an endemic fern of Taiwan, has been suspected to be a hybrid species. The aims of this study were to detect possible multiple origins of this species, determine the genetic variation in different populations, and clarify their lineages. One nuclear and three organellar DNA fragments were sequenced to determine parentage of this putative hybrid and to examine genetic differentiation among populations. Sequence data support the conclusion that *A. subreflexipinna* arose from the hybridization of *A. hasseltii* and *A. diffracta*, and the hybridization was uni-directional, i.e., based on the assumption of maternal inheritance in organellar DNA, the former was its maternal species while the latter was its paternal source. A convincing interpretation is that the female gametes of *A. hasseltii* gametophyte could be fertilized by the male gametes from apogamous *A. diffracta*. Unique nuclear alleles present in different populations of *A. subreflexipinna* and *A. hasseltii* demonstrated that hybridization occurred many times independently. The nuclear haplotypes present in *A. subreflexipinna* were subsets of those found in the parental species, and *A. subreflexipinna* always had lower haplotype diversity than *A. hasseltii* at sympatric sites. Our results show that any genetic variation of *A. subreflexipinna* came from its parents and that it maintains this significant genetic variability because of recurrent hybridization.

KEY WORDS.—*Acrorumohra diffracta*, *Acrorumohra hasseltii*, *Acrorumohra subreflexipinna*, hybridization, monilophytes, multiple origins

Hybridization followed by polyploidization is an important mechanism driving the formation of new lineages of ferns and other plants (Paun *et al.*, 2007). By means of diploidization processes, such as chromosomal rearrangements, intergenome recombination, and gene silencing, the genomic constitution of many extant taxa might be the outcome of ancient hybridization and polyploidy (Bowers *et al.*, 2003; De Bodt *et al.*, 2005; Haufler, 1987; Paun *et al.*, 2007). Hybridization events often begin these cycles and high chromosomal base number in ferns was achieved as the result of repeated cycles of

* corresponding author: e-mail: biofv017@ntnu.edu.tw

polyploidization (Haufler, 1987; Klekowski and Baker, 1966; Nakazato *et al.*, 2006).

Accessing the parentage of hybrids or allopolyploids is essential for understanding relationships within taxonomically complex groups. Although allozyme studies could provide tenable evidence to indicate the possible origin of hybrid-originated taxa, they have rarely been utilized to distinguish maternal lines from paternal ones. However, direct DNA evidence, such as nucleotide sequences and DNA fingerprints, can provide more informative insights into these evolutionary processes than enzymes. In most plants, organelle genomes are maternally inherited via female gametes while nuclear DNA is biparentally inherited (Soltis *et al.*, 1992). Comparing organellar DNA of hybrid taxa and their possible parents therefore could reveal the maternal origin (Gastony and Yatskievich, 1992; Vogel *et al.*, 1998) while comparing nuclear DNA of those taxa could show both putative parentages (Small *et al.*, 2004). In addition, any evolutionary trace, theoretically, would be deposited in nucleotide sequences and could be detected by DNA-based molecular technology. Unique local variation would be detectable if applicable DNA markers were chosen (Soltis *et al.*, 1992). DNA markers containing non-coding regions have been shown to be the best choice to reconstruct genealogies of hybrid and parental populations (Small *et al.*, 2004; Xiang *et al.*, 2000).

Studies of north temperate ferns have clearly indicated the contribution of hybridization and polyploidization to fern evolution (Barrington *et al*,. 1989; Bennert *et al.*, 2005; Pintér *et al.*, 2002; Wagner, 1973; Werth *et al.*, 1985). Out of the 420 species of lycophytes and ferns that grow in North America, nearly 20% are of hybrid origin (Flora of North America Editorial Committee, 1993), and reticulate networks and ploidy levels of most taxonomically complex groups have been well studied (Barrington, 1986; Stein and Barrington, 1990; Wagner, 1954, 1962, 1973; Xiang *et al.*, 2000). However, only a few ferns from other regions have received taxonomic attention like those in Europe and North America (e.g., Barrington, 1990; Ebihara *et al.*, 2005; Takamiya *et al.*, 2001; Terada and Takamiya, 2006). Some hybrid ferns have been recorded from Taiwan (Holttum and Edwards, 1986; Kuo, 1988, 1990; Miyamoto and Nakamura, 1983), but until now, no direct evidence has been reported to test and verify their parentage.

Acrorumohra is a small genus with about seven species distributed in Eastern and Southeastern Asia. This genus has an intermediate morphology between *Dryopteris* and *Arachniodes*; therefore, species of *Acrorumohra* were once treated in these genera. However, *Acrorumohra* was treated as an independent genus in the Flora of Taiwan (Shieh *et al.*, 1994) and Flora Reipublicae Popularis Sinicae (Hsieh, 2000) based on the presence of the zigzag rachis and anadromous pinnules of pinnae. *Acrorumohra subreflexipinna* (M. Ogata) H. Ito, an endemic species of Taiwan, produces shriveled and abortive spores and has an intermediate morphology between *A. hasseltii* (Blume) Ching and *A. diffracta* (Baker) H. Ito. Given its morphological characteristics, *A. subreflexipinna* has been suspected as a hybrid of these two species (Moore, 2000). Moreover, the fact that *A. subreflexipinna* always grows

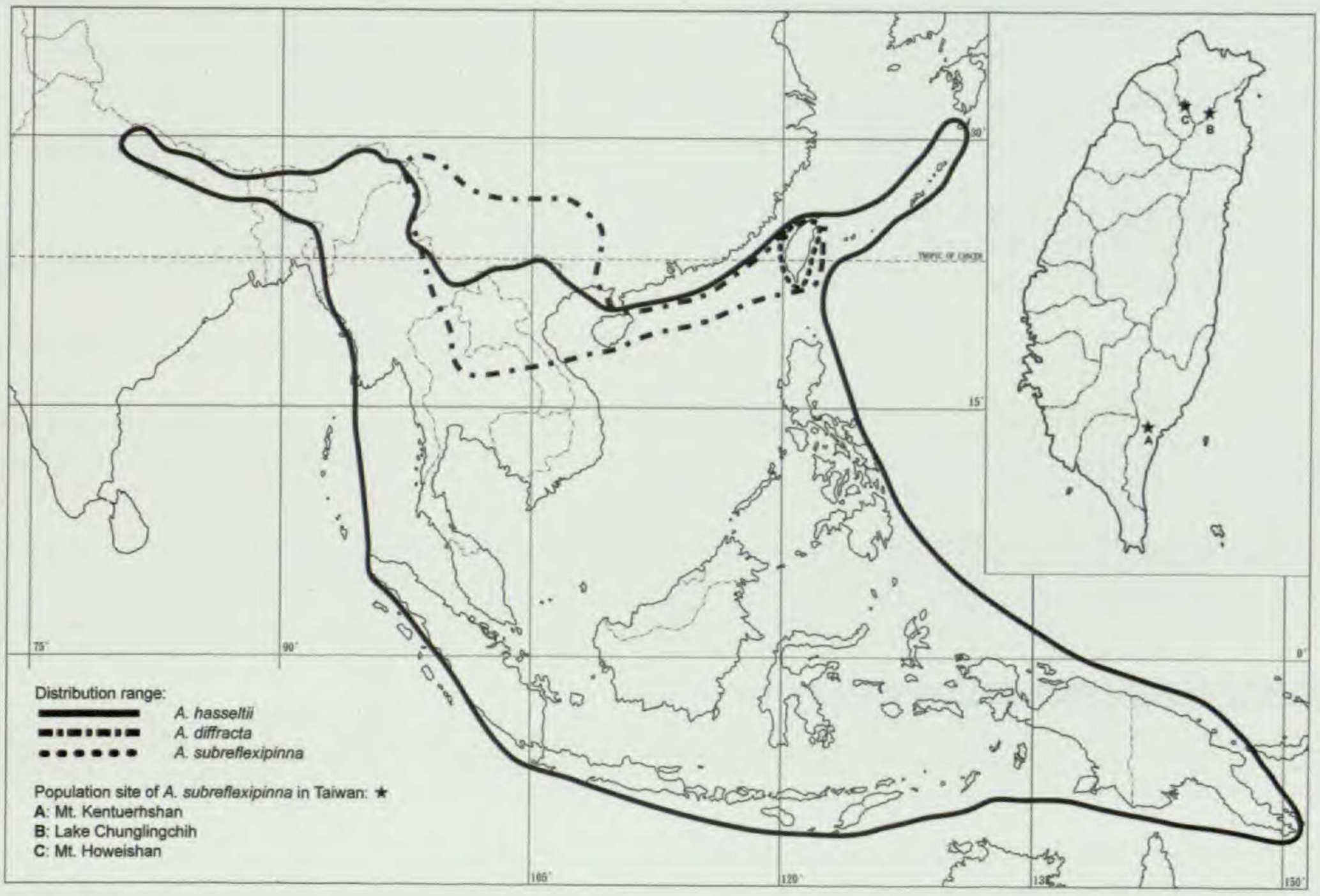

FIG. 1. Distribution of *Acrorumohra subreflexipinna*, *A. hasseltii*, and *A. diffracta*, and collection sites in this study.

sympatrically with the later two species reinforces the reasonable hypothesis of its hybrid origin. The narrowly defined genus '*Acrorumohra*' was followed and the scientific name '*A. subreflexipinna*' is used throughout the study, although palynological and unpublished breeding data indicates it a sterile F1 hybrid. In this study, chloroplast, mitochondria and nucleus DNA markers were used to identify the parentage of this suspected hybrid. Furthermore, the hypothesis that hybrid populations in Taiwan each originated independently was tested. In addition to haplotype comparison, genetic variation in different populations was determined to clarify lineage relationships.

MATERIALS AND METHODS

Plants of *Acrorumohra subreflexipinna* were sampled from three sites in Taiwan: Mt. Howeishan, Lake Chunglingchih and Mt. Kentuerhshan (Fig. 1). Leaf tissue of four to 11 individuals per population was collected for molecular analyses. Ten individuals of the two putative parent species, *A. hasseltii* and *A. diffracta*, were also sampled in each sympatric site (Table 1). Two plants of *Dryopteris polita* Rosenst. were also sampled to detect any possible parental relationship because based on phylogenetic analysis of a chloroplast *trnS-rps4* data set, *D. polita* and *A. hasseltii* are sister species (Li and Lu, 2006).

Two chloroplast intergenic spacers (*trnL-trnF* and *trnS-rps4* IGS) and one mitochondrial intron (*nad5* intron 2), which have been frequently used for

TABLE 1. Voucher information, quantity of sample and GenBank accession numbers for taxa used in molecular analysis of this study.

Taxon	Locality (population code)/sample no.	Cloned sample no./no. of clones	Voucher/deposited herbarium	DNA region/GenBank accession no.
Acrorumohra diffracta	1. Mt. Kentuerhshan (A)/10	3/15	*Chang 6316*/TNU	*trnL-trnF*/EU797681
				trnS-rps4/EU797685
				nad5 intron 2/EU797695
				pgiC intron 14–15/EU797705
	2. Lake Chunglingchih (B) /10	3/15	*Chang 6538*/TAIE	*trnL-trnF*/EU797682
				trnS-rps4/EU797686
				nad5 intron 2/EU797696
				pgiC intron 14–15/EU797706
	3. Mt. Howeishan (C) /10	3/15	*Chang 6667*/TAIE	*trnL-trnF*/EU797683
				trnS-rps4/EU797687
				nad5 intron 2/EU797697
				pgiC intron 14–15/EU797707
Acrorumohra hasseltii	1. Mt. Kentuerhshan (A) /10	3/15	*Chang 6319*/TNU	*trnL-trnF*/EU797679
				trnS-rps4/EU797691
				nad5 intron 2/EU797701
			Chang 6320/TNU	*pgiC* intron 14–15/EU797708
				pgiC intron 14–15/EU797709
	2. Lake Chunglingchih (B) /10	5/29	*Chang 6539*/TAIE	*trnL-trnF*/EU797680
				trnS-rps4/EU797692
				nad5 intron 2/EU797702
			Chang 6544/TAIE	*pgiC* intron 14–15/EU797710
			Chang 6540/TAIE	*pgiC* intron 14–15/EU797711
				pgiC intron 14–15/EU797712
	3. Mt. Howeishan (C) /10	4/23	*Chang 6673*/TAIE	*trnL-trnF*/EU797677
				trnS-rps4/EU797693
				nad5 intron 2/EU797703
			Chang 6674/TAIE	*pgiC* intron 14–15/EU797713
			Chang 6675/TAIE	*pgiC* intron 14–15/EU797714
				pgiC intron 14–15/EU797715

TABLE 1. Continued.

Taxon	Locality (population code)/sample no.	Cloned sample no./no. of clones	Voucher/deposited herbarium	DNA region/GenBank accession no.
Acrorumohra subreflexipinna	1. Mt. Kentuerhshan (A)/5	5/52	*Chang 6317*/TNU	*trnL-trnF*/EU797678 *trnS-rps4*/EU797688 *nad5* intron 2/EU797698
			Chang 6322/TNU	*pgiC* intron 14–15/EU797720 *pgiC* intron 14–15/EU797716 *pgiC* intron 14–15/EU797719
	2. Lake Chunglingchih (B)/4	4/37	*Chang 6541*/TAIE	*trnL-trnF*/EU797675 *trnS-rps4*/EU797689 *nad5* intron 2/EU797699
			Chang 6542/TAIE	*pgiC* intron 14–15/EU797717 *pgiC* intron 14–15/EU797721 *pgiC* intron 14–15/EU797723
	3. Mt. Howeishan (C)/11	11/91	*Chang 6671*/TAIE	*trnL-trnF*/EU797676 *trnS-rps4*/EU797690 *nad5* intron 2/EU797700 *pgiC* intron 14–15/EU797718
			Chang 6672/TAIE	*pgiC* intron 14–15/EU797722 *pgiC* intron 14–15/EU797724
Dryopteris polita	Lienhuachih/2	2/10	*Chang 6903*/TAIE	*trnL-trnF*/EU797684 *trnS-rps4*/EU797694 *nad5* intron 2/EU797704 *pgiC* intron 14–15/EU797725

phylogenetic analysis at lower taxonomic levels, were employed to reveal maternal history, while introns of the single-copy nuclear gene *pgiC* (including introns 14 and 15, and exon 15) were used to observe bi-parental inheritance. These sequences were chosen because of their significant phylogenetic information relative to other fragments and the availability of usable primers (Ishikawa *et al.*, 2002; Nadot *et al.*, 1995; Smith and Cranfill, 2002; Vangerow *et al.*, 1999).

Dry or fresh tissues of young leaves were homogenized with liquid nitrogen. Genomic DNA was extracted from ca. 100 mg of leaf tissue by using a Plant Genomic DNA Mini Kit (Viogene, USA). The PCR amplification of all segments was performed in an ABI thermocycler (9700). Primers for *trnL-trnF* IGS, trnLF-11 5′- GCG CAA GTT GCG GTA GAA CGA-3′ and trnLF-12 5′-CTG CTC TAC CGA CTG AG CTA-3′, were modifications of those utilized by Taberlet *et al.* (1991). The primers tsr4-f/tsr4-r 5′-CCC GCA AAG CTT AGT GAT CA-3′/ 5′-CCG AGG GTT CGA ATC CCT C-3′, nadh2-f/nadh2-r 5′-GGG GCT ATA TCG CCA TCC-3′/5′-CCG CAC GTG CAA GTT TCC-3′, and pgiC-14fA/pgiC-16rA 5′-GTG CTT CTG GGT CTT TTG AG-3′/5′-GTT GTC CAT TAG TTC CAG GT-3′ were developed for this study referring to Smith and Cranfill (2002), Vangerow *et al.* (1999) and Ishikawa *et al.* (2002), respectively. PCR reactions were carried out in 20 μL reactions containing 2 μL unstandardized template DNA, 0.2 mmol/L of each dNTP, 0.8 units of Taq polymerase (ABgene, USA) and 6.25 pmol each of the forward and reverse primers, and programmed for 5 min at 95°C, 35 cycles of 1 min at 95°C, 1 min at annealing temperature and 2 min at 72°C, followed by a 8 min extension at 72°C. The annealing temperature was 59°C in amplifying the chloroplast *trnL-trnF* IGS and the mitochondrial *nad5* intron 2, and 52°C in amplifying the chloroplast *trnS-rps4* fragment. When amplifying nuclear *pgiC* intron 14–15 segment, annealing was performed at 57°C for the first 3 cycles, at 55°C for the next 3 and at 54°C for the final 29. PCR products were directly sequenced, using one amplification primer, on an ABI 373A automated sequencer (Applied Biosystems, USA) with the Taq Dye Dideoxy Terminator Cycle Sequencing Kit (Applied Biosystems). For the electrophoresed bands with lengths greater than 500 bp, sequences were determined in both directions. Additionally, *pgiC* intron 14–15 segments of all *A. subreflexipinna* samples and 3–5 samples in each population of *A. hasseltii*and *A. diffracta* were cloned. The PCR products of the nuclear segment were purified by electrophoresis using 1× TAE buffer on a 1.2% agarose gel. Electrophoresed bands were cut and eluted using the Gel-M gel extraction system (Viogene). Purified nuclear DNA was cloned with the yT&A cloning kit (Yeastern Biotech, Taiwan) following the manufacturer's protocol. Five to eight colonies were chosen to perform colony PCR using TA-F forward and TA-R reverse primers (Yeastern Biotech). Purified nuclear DNA was sequenced with M13 universal and reverse primers which are located on the DH5α vector termination site. When any different haplotype was detected, repeated PCR reactions using a different *Taq* polymerase (Genomics, Taiwan) or using DNA from another three colonies were chosen to check whether it was a real variant or not. All sequences were deposited in the GenBank nucleotide

sequence database, and accession numbers and their corresponding DNA regions are listed in Table 1.

The sequences were aligned by BioEdit 7.0 and manual correction, and compared with nucleotide sequences available through GenBank to determine their boundaries of coding region. Haplotypes were named after the first letter of the specific epithet, and followed by a lowercase letter and number to designate different, minor haplotypes (those differing from their corresponding major haplotype at only one base) of *A. hasseltii*. Genetic diversity at population and species levels was estimated with the software package DNA Sequence Polymorphism (DnaSP 4.20.2, Rozas *et al.*, 2003). The haplotype diversity (h) and nucleotide diversity (π) of these three populations were calculated separately and totally. Genetic differentiation (γ_{ST}, Nei, 1982) among these three populations and between pairs of populations was also calculated by this package. γ_{st}, but not F_{ST} or N_{ST}, was used because the three sampled populations were the only ones of interest (Lynch and Crease, 1990). Because no variation was detected in the nuclear sequences of *A. diffracta*, only the *A. hasseltii* haplotypes cloned from *A. subreflexipinna* were used when analyzing genetic diversity and differentiation among the populations of *A. subreflexipinna*. Haplotypes of *A. hasseltii* and *A. subreflexipinna* were identified and coded by direct sequence comparison, and unrooted haplotype networks were constructed with the program TCS 1.21 (Clement *et al.*, 2000).

Results

Total aligned length and GC content of the sequences of nuclear *pgiC* intron 14–15, chloroplast *trnL-trnF* IGS and *trnS-rps4* IGS, and mitochondrial *nad5* intron 2 were 725 bp/37.8%, 268 bp/34.9%, 374 bp/36.5%, and 728 bp/52.6%, respectively. Low GC content of chloroplast segments agreed with the AT-rich property of most non-coding spacers (Graur and Li, 2000).

In the chloroplast and mitochondria segments, all 50 individuals of *A. subreflexipinna* and *A. hasseltii* had the same nucleotide sequences but were different from those of *A. diffracta* and *D. polita* (Tables 2–4). In the nuclear *pgiC* intron 14–15 sequences, *A. subreflexipinna* possessed both the *A. hasseltii* and the *A. diffracta* haplotypes (Table 5) but not that of *D. polita* (data not shown). *pgiC* intron 14–15 sequences of *A. diffracta* from the three populations were all the same (haplotype 'D'), but those of *A. hasseltii* and *A. subreflexipinna* in each population had two to three haplotypes (Tables 5 and 6). There were two major (Ha and Hb) and another three minor haplotypes (Ha1, Hb1 and Hb2) found in *A. hasseltii* (Table 5). These minor haplotypes differ from their corresponding major haplotypes at only one base, and were found in the three populations respectively. In total, five and four haplotypes were found in *A. hasseltii* and *A. subreflexipinna*, respectively.

When calculating haplotype diversity (h) and nucleotide diversity (π) (Table 6), the haplotype "D" was, *a priori*, removed from the genetic pool of *A. subreflexipinna* to avoid interference in comparison with that of *A. hasseltii*. In *A. hasseltii*, haplotype diversity (h) among these three populations ranged

TABLE 2. The variable nucleotide sites (indel & base substitution) of chloroplast *trnL-trnF* intergenic spacer sequences. Columns shaded are sites identical to the hybrid sequences.

	Variable sites																	
	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	2	2
Species	0	1	2	2	2	2	4	6	0	4	5	5	6	8	9	9	1	3
	7	9	0	1	2	3	9	8	5	2	1	6	6	8	8	9	6	2
A. diffracta	G	-	-	-	-	-	G	A	C	T	C	A	T	C	G	C	-	C
A. subreflexipinna	A	-	-	-	-	-	A	G	T	A	C	A	C	A	A	T	-	T
A. hasseltii	A	-	-	-	-	-	A	G	T	A	C	A	C	A	A	T	-	T
D. polita	G	T	A	G	T	T	A	G	T	A	T	G	C	G	A	T	A	C

from 0.533 to 0.689, and it was 0.724 at the species level. In *A. subreflexipinna*, haplotype diversity among populations ranged from 0.400 to 0.667, and it was 0.674 at the species level. Nucleotide diversity (π) among the three populations of *A. hasseltii* ranged from 0.00074 to 0.00446, and it was 0.00485 at the species level. In *A. subreflexipinna*, nucleotide diversity among populations ranged from 0.00055 to 0.00553, and it was 0.00466 at the species level.

For the nuclear *pgiC* segment of *A. hasseltii* and *A. subreflexipinna*, the Ha haplotype could be clearly distinguished from Hb by six sites with different base pairs and two indel sites (Table 5; Fig. 2). The Ha haplotype has a minor type (Ha1) with a single base difference. This minor haplotype is found only in the Mt. Kentuerhshan population of *A. hasseltii* and *A. subreflexipinna* (Fig. 2). On the other hand, the Hb haplotype has two single base change minors (Hb1 and Hb2) occurring respectively in Lake Chunglingchih and Mt. Howeishan populations of *A. hasseltii* (Fig. 2(i)). In *A. subreflexipinna*, genetic variation among different individuals and/or populations directly came from different haplotypes of *A. hasseltii*. For example, in *A. subreflexipinna* of Mt. Kentuerhshan, except for the haplotype that was identical to *A. diffracta*, there were two haplotypes (Ha and Ha1) that were also found in *A. hasseltii* of the sympatric site. Nuclear haplotypes of *A. hasseltii* in Mt. Howeishan and Lake Chunglingchih were identical except for the two minors (Hb1 and Hb2). However, only one major haplotype (Ha) was found in *A. hasseltii* and *A. subreflexipinna* of Mt. Kentuerhshan. The Hb and derivatively minor haplotypes were found neither in *A. hasseltii* nor *A. subreflexipinna* of Mt. Kentuerhshan.

TABLE 3. The variable nucleotide sites (indel & base substitution) of chloroplast *trnS-rps4* intergenic spacer sequences. Columns shaded are sites identical to the hybrid sequences.

	Variable sites																						
	0	0	0	0	0	1	1	1	1	2	2	2	2	2	2	2	2	2	3	3	3	3
Species	3	7	7	8	8	4	4	4	9	0	1	1	1	3	4	5	5	6	2	4	4	7
	9	2	6	2	4	0	1	2	3	8	2	4	7	0	1	2	8	8	7	7	8	3
A. diffracta	A	T	G	G	G	-	-	T	A	T	A	G	C	C	C	T	A	C	C	G	A	T
A. subreflexipinna	G	G	A	G	A	-	-	C	G	C	C	G	C	T	T	C	C	C	T	G	G	C
A. hasseltii	G	G	A	G	A	-	-	C	G	C	C	G	C	T	T	C	C	C	T	G	G	C
D. polita	A	G	G	T	G	T	T	T	G	T	C	C	T	C	C	C	C	T	T	A	G	T

The level of divergence among the three populations could not be revealed by the organellar fragments because only one haplotype was detected in each species (Tables 2–4). For nuclear *pgiC* intron 14–15 sequences, however, DnaSP analysis revealed high levels of genetic differentiation among three populations of *A. hasseltii* and *A. subreflexipinna* (γ_{ST} = 0.44377 and γ_{ST} = 0.26399; Table 7). Additionally, higher levels of genetic differentiation were also detected between northern and southeastern populations (A–B and A–C; Table 7) of these two species while little differentiation was found between those northern two (B–C; Table 7). For this same fragment, on the other hand, *A. diffracta* had only one haplotype and indicated no pattern of population structure.

DISCUSSION

Hybridization and parentage.—Similar to the traditional circumscription of species, hybrid species and hybrid parentage are usually postulated initially based on morphological characters and degree of spore/pollen abortion (Barrington, 1989, 1990). *Acrorumohra subreflexipinna* is suspected as a natural hybrid between *A. hasseltii* and *A. diffracta* (Moore, 2000) because *A. subreflexipinna* has abortive spores and intermediate morphology between *A. hasseltii* and *A. diffracta*, and occurs sympatrically with these two species. In addition, *A. subreflexipinna*'s spores show no germination, but those of *A. hasseltii* and *A. diffracta* germinate at a rate of more than 80% (unpublished data). Therefore, *A. subreflexipinna* appears to be a sterile F1 hybrid.

TABLE 4. The variable nucleotide sites (indel & base substitution) of mitochondrion *nad5* intron 2 sequences. Columns shaded are sites identical to the hybrid sequences.

Species	Variable sites														
	2	2	2	2	2	3	3	3	3	4	7	7	7	7	7
	1	1	1	1	1	7	7	8	8	1	0	0	0	0	1
	3	4	5	6	7	8	9	0	1	1	6	7	8	9	0
A. diffracta	T	G	G	T	T	T	-	T	T	T	C	C	G	C	T
A. subreflexipinna	-	-	-	-	-	C	A	C	C	T	-	-	-	-	-
A. hasseltii	-	-	-	-	-	C	A	C	C	T	-	-	-	-	-
D. polita	T	G	G	T	T	T	-	T	T	-	-	-	-	-	-

Acrorumohra subreflexipinna with its perennial habit, however, could occupy an original habitat for a long time despite of all spores being sterile. Repeated hybridization where the putative parents sympatrically exist might also replenish the stock of this hybrid.

Organelle genomes are generally maternally inherited in monilophytes (Gastony and Yatskievich, 1992; Vogel *et al.*, 1998). The assumption that chloroplast and mitochondria are maternally inherited is adopted through this study. All organellar sequence data indicated that *A. hasseltii* was the maternal parent of *A. subreflexipinna*. Nuclear *pgiC* sequences indicated that *A. diffracta* was the other genome donor of this hybrid.

In addition to the three taxa of *Acrorumohra* discussed here, another species, *A. yoroii* (Seriz.) Shieh, was reported in the second edition of Flora of Taiwan (Shieh *et al.*, 1994). In Taiwan, it grows in high montane regions and never sympatrically with other three taxa of *Acrorumohra*. Samples of that species were also collected from Taiwan and sequenced. It has organellar and nuclear sequences different from those of *A. subreflexipinna*, and phylogenetic analysis indicates a distant relationship between them (data not shown).

There are three other species of this narrowly defined genus. *Acrorumohra dissecta* Ching ex Hsieh is distributed in a few locations of southwestern China, and *A. obtusissima* (Mett. ex Kuhn) Ching and *A. undulata* (Bedd.) Ching are distributed throughout Sri Lanka. Though we cannot reject the hypothesis, the possibility of these species contributing to the formation of this hybrid is extremely low because of their restricted habitats and disjunct distribution from this hybrid.

TABLE 5. The haplotype and variable nucleotide sites (indel & base substitution) of nuclear *pgiC* intron 14–15 sequences. Columns shaded are sites different from those of other populations.

Site[a]	Species	Haplotype code[b]	Frequency	Variable sites: 0 4 8	0 6 3	0 9 1	1 2 2	1 4 4	1 6 9	2 2 6	2 4 7	2 5 3	2 5 4	2 6 6	3 0 8	3 4 4	3 5 0	3 8 1	4 5 0	4 5 1	4 6 5	4 7 8	5 2 2	5 2 3	5 3 4	5 7 3	6 0 9	6 1 0	6 6 4	6 7 0
A	*A. diffracta*	D	10	C	G	A	G	C	C	T	A	T	G	C	T	A	G	A	C	A	C	T	-	-	A	G	C	A	T	T
		D	5	C	G	A	G	C	C	T	A	T	G	C	T	A	G	A	C	A	C	T	-	-	A	G	C	A	T	T
	A. subreflexipinna	Ha	1	T	G	T	C	T	T	C	G	C	T	C	C	A	A	G	-	-	C	C	C	T	G	A	T	G	C	G
		Ha1	4	T	G	T	C	T	T	C	G	C	T	**T**	C	A	A	G	-	-	C	C	C	T	G	A	T	G	C	G
	A. hasseltii	Ha	6	T	G	T	C	T	T	C	G	C	T	C	C	A	A	G	-	-	C	C	C	T	G	A	T	G	C	G
		Ha1	4	T	G	T	C	T	T	C	G	C	T	**T**	C	A	A	G	-	-	C	C	C	T	G	A	T	G	C	G
B	*A. diffracta*	D	10	C	G	A	G	C	C	T	A	T	G	C	T	A	G	A	C	A	C	T	-	-	A	G	C	A	T	T
		D	4	C	G	A	G	C	C	T	A	T	G	C	T	A	G	A	C	A	C	T	-	-	A	G	C	A	T	T
	A. subreflexipinna	Ha	2	T	G	T	C	T	T	C	G	C	T	C	C	A	A	G	-	-	C	C	C	T	G	A	T	G	C	G
		Hb	2	T	**A**	T	C	**C**	T	C	G	C	T	C	C	A	A	**A**	**C**	**A**	**G**	C	C	T	G	A	**C**	G	C	**T**
		Ha	2	T	G	T	C	T	T	C	G	C	T	C	C	A	A	G	-	-	C	C	C	T	G	A	T	G	C	G
	A. hasseltii	Hb	5	T	**A**	T	C	**C**	T	C	G	C	T	C	C	A	A	**A**	**C**	**A**	**G**	C	C	T	G	A	**C**	G	C	**T**
		Hb1	3	T	**A**	T	C	**C**	T	C	G	C	T	C	C	**G**	A	**A**	**C**	**A**	**G**	C	C	T	G	A	**C**	G	C	**T**
C	*A. diffracta*	D	10	C	G	A	G	C	C	T	A	T	G	C	T	A	G	A	C	A	C	T	-	-	A	G	C	A	T	T
		D	11	C	G	A	G	C	C	T	A	T	G	C	T	A	G	A	C	A	C	T	-	-	A	G	C	A	T	T
	A. subreflexipinna	Ha	5	T	G	T	C	T	T	C	G	C	T	C	C	A	A	G	-	-	C	C	C	T	G	A	T	G	C	G
		Hb	6	T	**A**	T	C	**C**	T	C	G	C	T	C	C	A	A	**A**	**C**	**A**	**G**	C	C	T	G	A	**C**	G	C	**T**
		Ha	4	T	G	T	C	T	T	C	G	C	T	C	C	A	A	G	-	-	C	C	C	T	G	A	T	G	C	G
	A. hasseltii	Hb	5	T	**A**	T	C	**C**	T	C	G	C	T	C	C	A	A	**A**	**C**	**A**	**G**	C	C	T	G	A	**C**	G	C	**T**
		Hb2	1	T	**G**	T	C	**C**	T	C	G	C	T	C	C	A	A	**A**	**C**	**A**	**G**	C	C	T	G	A	**C**	G	C	**T**

[a] A: Mt. Kentuerhshan, B: Lake Chunglingchih, C: Mt. Howeishan.

[b] First letter designated to different haplotypes originated from different species; D: *A. diffracta*, H: *A. hasseltii*. Lowercase letter and number designated to different minor haplotypes of *A. hasseltii*.

TABLE 6. Number of haplotypes, estimates of haplotype diversity (h) and nucleotide diversity (π) of *A. hasseltii* and *A. subreflexipinna*. The *A. diffracta* haplotype 'D' cloned from *A. subreflexipinna* were excluded prior to this analysis.

Populations	Species	No. of individuals	No. of haplotypes	Haplotype diversity (h)	Nucleotide diversity (π)
Total (A+B+C)	*A. hasseltii*	30	5	0.724	0.00485
	A. subreflexipinna	20	3	0.674	0.00466
Mt. Kentuerhshan (A)	*A. hasseltii*	10	2	0.533	0.00074
	A. subreflexipinna	5	2	0.400	0.00055
Lake Chunglingchih (B)	*A. hasseltii*	10	3	0.689	0.00360
	A. subreflexipinna	4	2	0.667	0.00553
Mt. Howeishan (C)	*A. hasseltii*	10	3	0.644	0.00446
	A. subreflexipinna	11	2	0.545	0.00453

Although the phylogenetic analysis based on chloroplast *trnS-rps4* IGS sequence show a sister-group relationship between *D. polita* and *A. hasseltii* (Li and Lu, 2006; our unpublished data), this study reveals that *A. subreflexipinna* has sequences different from those of *D. polita* in both organellar (Tables 2–4) and nuclear (data not shown) genomes. Therefore, *Dryopteris polita* did not contribute to the formation of this hybrid. These molecular data plus morphological and ecological information explicitly suggest that *A. subreflexipinna* arose through hybridization of *A. hasseltii* and *A. diffracta*, and that the former was its putative maternal parent while the latter was its paternal source.

Acrorumohra hasseltii is distributed in tropical Asia, including Java, Borneo, Thailand, Nepal, East Himalayas, Vietnam, Hainan, Taiwan and southern Japan (Fig. 1). The range of *A. diffracta* overlaps with *A. hasseltii* in the northern portion of the range of *A. hasseltii*, i.e., East Himalayas, northern Thailand, Vietnam, Hainan and Taiwan (Fig. 1). Except for southwestern China, the geographic range of *A. diffracta* almost completely overlaps with that of *A. hasseltii*. However, *A. subreflexipinna* has only been reported from Taiwan. It is suspected that *A. subreflexipinna* might be established at some sites across this widely overlapping range of these two putative parents but misidentified as *A. diffracta* because of their similar zigzag rachis. Careful recognition is needed to identify this hybrid in future field investigation where the range of these two species overlaps.

Gender bias in hybridization events has been demonstrated many times in plants (Emms *et al.*, 1996; Vogel *et al.*, 1998; Weiblen and Brehm, 1996; Xiang *et al.*, 2000). For reasons not entirely clear, the hybridization of *A. hasseltii* and *A. diffracta* in our study was absolutely biased, i.e., *A. hasseltii* always was the supplier of egg while *A. diffracta* was that of sperm. This phenomenon has also been found in other hybrid species (e.g., Arnold and Bennett, 1993; Peng and Chiang, 2000; Smith and Sytsma, 1990; Wendel *et al.*, 1991). In ferns, mating systems usually correlate with ploidy levels and could be a decisive factor in the nuclear-organellar combination pattern of parental genotypes in hybridization. In fact, *A. diffracta* was reported as a tetraploid (Tsai and Shieh, 1975)

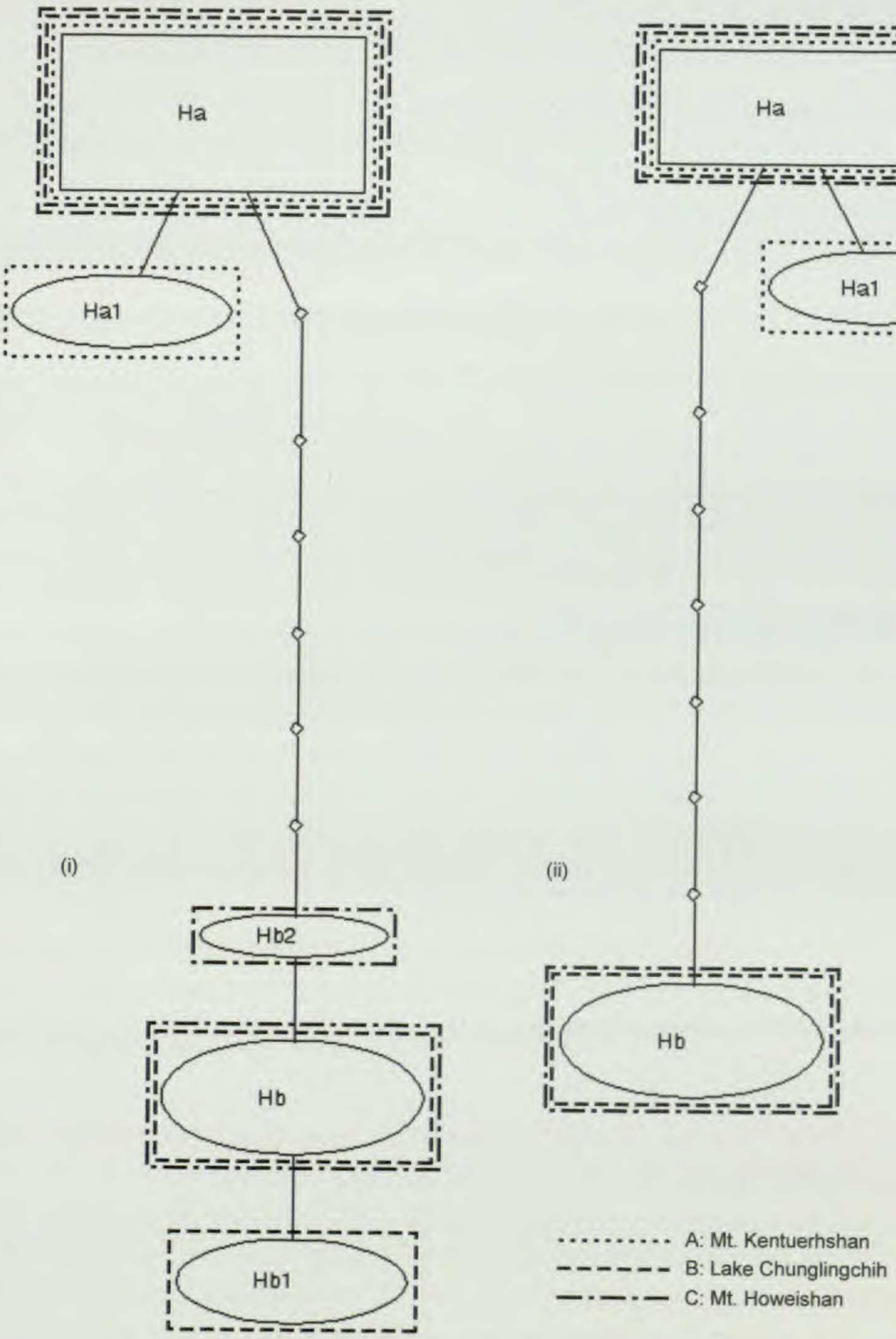

FIG. 2. *pgiC* intron 14–15 network for *Acrorumohra hasseltii* (i) and *A. subreflexipinna* (ii). Letters and number in the boxes or circles are haplotype codes. Dashed boxes indicate site(s) in which the haplotype is detected. The little rhombuses indicate the hypothetical haplotypes. Each line between haplotypes represents a mutational step.

and *A. hasseltii* a diploid (Iwatsuki, 1995), and our observation showed that *A. hasseltii* has 64 spores per sporangium but *A. diffracta* has 32 spores (unpublished data). These agree with the general rule that a sexual species usually has 64 spores per sporangium whereas there are typically 32 spores in an apogamous species. According to the Dopp-Manton Scheme and Braithwaite modes of reproduction (Raghavan, 1989), *Acrorumohra diffracta*

TABLE 7. Genetic differentiation between pairwise and among all populations of *A. hasseltii* and *A. subreflexipinna*.

Species	Genetic differentiation among all populations (γ_{ST})	Genetic differentiation between pairwise populations (γ_{ST})[a]		
		A–B	A–C	B–C
A. hasseltii	0.44377	0.60815	0.40303	0.06301
A. subreflexipinna	0.26399	0.40000	0.31460	0.00162

[a]A: Mt. Kentuerhshan, B: Lake Chunglingchih, C: Mt. Howeishan.

might be an obligate apogamous species with functional antheridia. These observations and molecular evidence lead to a convincing hypothesis for unidirectional hybridization that sexual *A. hasseltii* could adopt sperm from the apogamous tetraploid, *A. diffracta*. Additionally, a lack of heterozygosity of the *A. hasseltii* genotypes in this hybrid further supports that *A. hasseltii* was the haploid female gamete donor in these hybridization events. In this case, because fertile sperm were liberated from an apogamous, tetraploid gametophyte, *A. subreflexipinna* should be a pentaploid. C-value results based on flow cytometry show that *A. subreflexipinna* has the highest ploidy level among these taxa (unpublished data), which confirms this hypothesis. Further observations on chromosome counts and the mating system of *A. subreflexipinna* and both parents in the future would provide more direct evidence.

Multiple independent origins.—Although *A. subreflexipinna* has a larger body size than both putative parental species, it produces no fertile spores, and its small population size and rare occurrence strongly suggests it is a product of occasional hybridization event(s). Both cpDNA and mtDNA fragments of populations of *A. hasseltii* and *A. subreflexipinna* were identical in sequence. Single direction hybridization and allopolyploidization may produce cytoplasmically uniform hybrids or allopolyploids despite distinct origins (Soltis *et al.*, 1992). The variation in cpDNA and mtDNA from the taxa of this study was uninformative regarding multiple origins of *A. subreflexipinna*. Nuclear *pgiC* sequences, however, revealed different haplotypes and genetic differentiation among populations of *A. hasseltii* and *A. subreflexipinna*. Southern and northern populations of *A. hasseltii* had distinct and unique genotypes, and the genetic uniqueness was transmitted to their hybrid offspring (Table 5; Fig. 2). In both *A. hasseltii* and *A. subreflexipinna*, there were nine variable sites that had different bases in southern and northern populations (Table 5). Haplotype Ha1 was found in *A. hasseltii* of Mt. Kentuerhshan and was present in *A. subreflexipinna* in the same location. On the other hand, major haplotype Hb was only found in the northern populations of both species but not in the southern ones (Fig. 2). These are direct indicators of multiple independent origins of *A. subreflexipinna*.

There was no variation of organellar DNA fragments in populations of *A. hasseltii* and *A. subreflexipinna*, but high nucleotide diversity was found in the nuclear *pgiC* intron 14–15 of the same species. The fact that, usually,

evolutionary rates of nuclear DNA are faster than those of chloroplast and mitochondria in plants (Graur and Li, 2000) may provide a reasonable interpretation of this significant difference. This also indicates that nuclear DNA markers may be a better choice when analyzing population variation due to relatively short evolutionary time. In this case, geographical barriers might effectively hinder gene flow, causing geographical subdivision in nuclear DNA, but the time of isolation might not have been long enough to accumulate new mutations in organellar DNA.

Several studies indicate that multiple origins of hybrid and polyploid species are common, if not the rule, in plants (see Soltis *et al.*, 1992 and references therein). Genetic variation of the parental species could be incorporated into and preserved in hybrids and their derivative taxa by these processes (Arft and Ranker, 1998; Paun *et al.*, 2007; Peng and Chiang, 2000). This phenomenon was also found in this study. Unlike a small population usually having fixed alleles for most loci, populations of *A. subreflexipinna*, even when only four individuals were found in a population, have high haplotype diversity. Moreover, the results agree with our anticipation that in each collecting site there were fewer haplotypes and lower haplotype diversity of *A. subreflexipinna* than those of *A. hasseltii*. Because *A. subreflexipinna* is a sterile hybrid, any genetic variation should come from its parents. It is the recurrent hybridization of this hybrid that might maintain its significant genetic variability and provide operative materials for future evolution, i.e., polyploidization and subsequent fertilization.

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